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TITLE: GKLF as a Novel Target in Selenium Chemoprevention of Prostate Cancer

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CONTRACTING ORGANIZATION: Health Research Inc.
                                             Buffalo NY 14263

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14. ABSTRACT
The present study investigated the functional significance of the zinc finger transcription factor gut-enriched krüppel-like factor (GKLF) in mediating selenium action in the androgen receptor (AR)-null PC-3 human prostate cancer cells. We found that overexpression of GKLF enhances selenium inhibition of DNA synthesis and induction of apoptosis. Furthermore, knocking down the expression of GKLF greatly attenuates the growth suppressive and apoptosis inducing activities of selenium. Therefore, our data support an important role of GKLF induction in selenium action in the AR-null prostate cancer cells. However, we found that, in cells expressing a functional AR, the disruption of AR signaling is most likely more important than the induction of GKLF signaling for selenium action. Selenium treatment significantly decreases the expression of AR and AR-regulated genes implicated in prostate carcinogenesis (PSA, KLK2, ABCC4, DHCR24, and GUCY1A3) in five human prostate cancer cell lines irrespective of their AR genotype (wild-type vs. mutant) or sensitivity to androgen-stimulated growth. Transfection of AR in the androgen-dependent LNCaP cells weakens significantly the inhibitory effect of selenium on cell proliferation and AR target gene expression. Since the vast majority of prostate cancers, including those refractory to hormone therapy, express a functional AR, the disruption of AR signaling is probably more important for selenium action and more relevant to selenium chemoprevention of prostate cancer than the induction of GKLF.

15. SUBJECT TERMS
selenium, cancer chemoprevention, prostate cancer, GKLF transcription factor, androgen receptor

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Molecular Cancer Therapeutics, 4, 1047-1055, 2005.

A. INTRODUCTION:

This project was designed initially to investigate the role of the zinc finger transcription factor gut-enriched krüppel-like factor (GKLF) in contributing to the molecular effects of selenium in cancer chemoprevention. In the first annual progress report, we described the mechanistic basis for GKLF upregulation by selenium and the effect of GKLF overexpression on the growth of prostate cancer cells. Our data indicated a growth suppressive and pro-apoptotic function of GKLF in the androgen receptor (AR)-null PC-3 cells. However, the LNCaP cells, which contain a functional AR, responded to GKLF overexpression by inducing the expression of AR, and the effect of which predominated, leading to a modest stimulation of cell growth. We also found that selenium is able to markedly suppress AR expression. Exogenous expression of AR attenuated the growth suppressive activity of selenium, although accompanied by a significant increase in GKLF level. The data suggest that disruption of AR signaling is probably more important than the induction of GKLF signaling for selenium action in AR-expressing cells. Therefore, as approved by the DOD Prostate Cancer Research Program, we continued our GKLF study in the AR-null PC-3 cells, but shifted our research focus to selenium suppression of AR signaling in the androgen-responsive LNCaP cells.

B. BODY:

Results for Task 1 (To study whether transcriptional or post-transcriptional mechanism is responsible for mediating selenium upregulation of GKLF expression):

MSA induces GKLF mRNA level. Fig. 1 shows MSA upregulation of the GKLF transcript, as determined by real-time RT-PCR, during a 6 or 16 hr period in PC-3 and LNCaP cells. The increase in GKLF transcript occurred very quickly. There was about a 2-fold induction in the first two hours after treatment with 10 μM MSA in both cell lines. In LNCaP cells, the induction by MSA reached the maximum at this time point. In contrast, the magnitude of induction rose to ~3.5-fold at 3 hr in PC-3 cells, and remained at this level upon longer exposure.
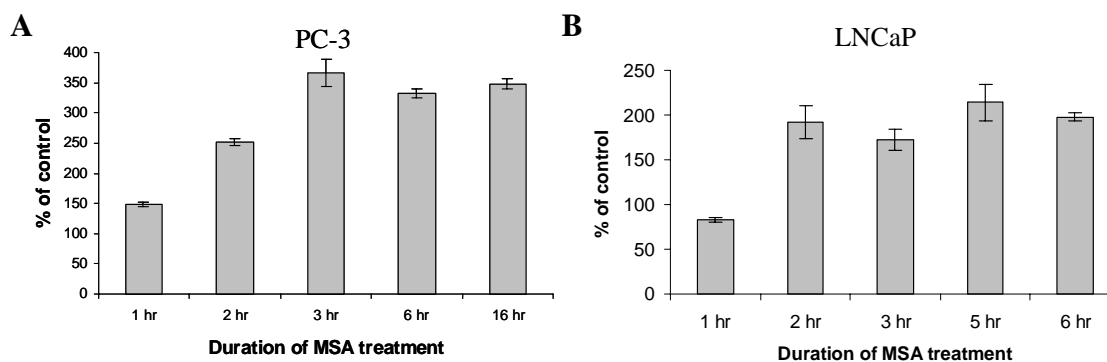

Fig. 1. Effect of MSA on GKLF mRNA expression as determined by real time RT-PCR analysis. The results are expressed as % of control; bars represent SEM. With the exception of the 1 hr data point in LNCaP cells, the remaining data points are statistically different (P < 0.01) from the untreated control.

MSA increases the stability of GKLF mRNA. We next performed an mRNA stability assay under the condition in which new RNA synthesis was blocked. Actinomycin D,

an RNA synthesis inhibitor, was added to the culture at the time of MSA treatment, and GKLF mRNA levels were followed in a 6-hr time course by real-time RT-PCR analysis. Since actinomycin D could be cytotoxic, we also monitored cell growth for a duration of up to 8 hr and did not observe cell death or significant growth inhibition during this period. Our results showed that treatment with MSA increased the stability of GKLF mRNA in both cell lines (Fig. 2).
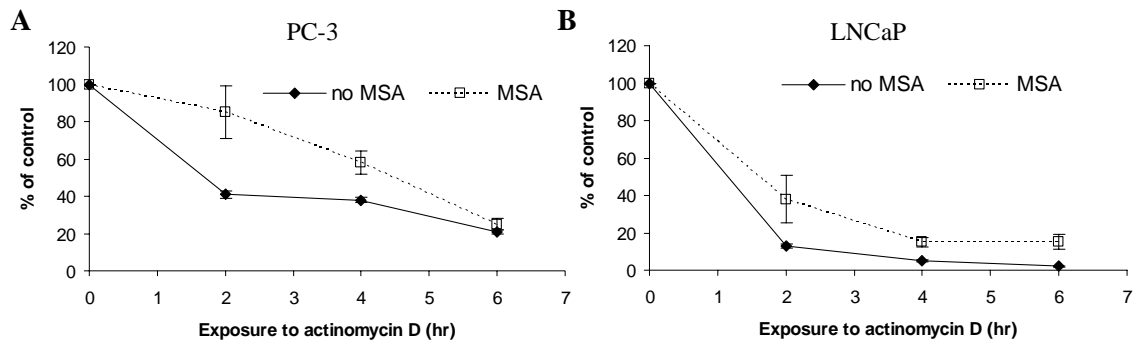

Fig. 2. Effect of MSA on GKLF mRNA stability. The results are expressed as % of control; bars represent SEM.

MSA does not change the activity of a 1 kb proximal GKLF promoter. In order to study the effect of MSA on GKLF promoter activity, a luciferase reporter gene construct containing about 1 kb fragment of the proximal promoter region of the GKLF gene (kindly provided by Dr. Vincent W. Yang at the Emory University) was transiently transfected into PC-3 and LNCaP cells. The luciferase reporter assay was carried out at 1 hr, 2 hr, 3 hr, 6 hr, or 16 hr after treatment with 10 μM MSA. As can be seen in Fig. 3, MSA treatment did not result in any significant change in the activity of this 1 kb GKLF promoter. The data indicate that either MSA does not affect GKLF mRNA transcription initiation, or the *cis* element(s) mediating MSA effect is not present in this 1 kb promoter region of the GKLF gene.
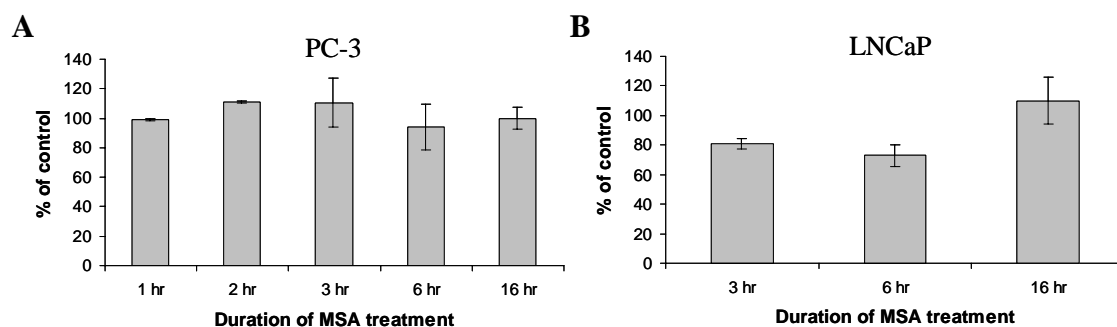

Fig. 3. Effect of MSA on GKLF-promoter activity. The results are expressed as % of control; bars represent SEM. None of the data points is statistically different (P < 0.01) from the untreated control.

Results for Task 2 (To determine the effect of GKLF overexpression on the growth of prostate cancer cells as well as selenium growth inhibition):

Overexpression of GKLF enhances MSA inhibition of DNA synthesis in PC-3 cells. In an attempt to investigate the biological consequence of GKLF induction by MSA, we transiently transfected PC-3 cells with a GKLF expression construct, pcDNA3.1/His B-GKLF (kindly

provided by Dr. Anil K. Rustgi at the University of Pennsylvania) and assessed the effect of GKLF overexpression on MSA growth inhibition. Cell proliferation was quantified with the combined use of the cell viability detection reagent WST-1 (Roche) and a BrdU ELISA system (Roche), which measures BrdU incorporation during DNA synthesis. The BrdU ELISA data were normalized by the WST-1 results to correct for the differences in cell number. As shown in Fig. 4, after exposure to 5 µM MSA for 16 hr, DNA synthesis was suppressed by 37% in cells transfected with the mock vector. The suppression was significantly greater (50%, $P < 0.05$) in cells overexpressing GKLF.
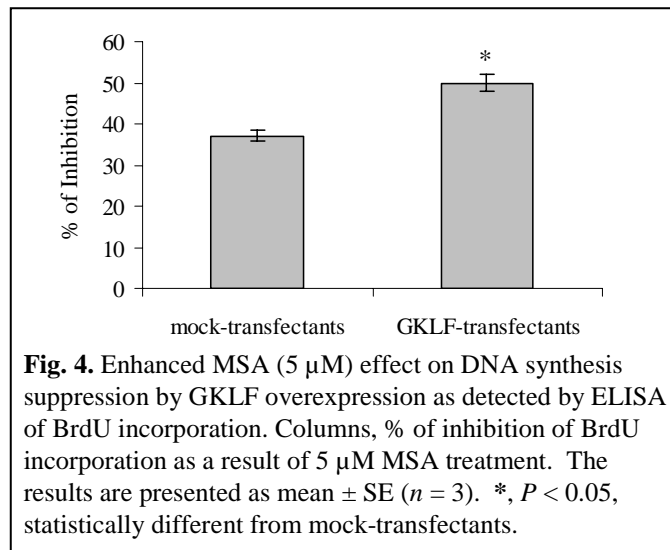

Fig. 4. Enhanced MSA (5 µM) effect on DNA synthesis suppression by GKLF overexpression as detected by ELISA of BrdU incorporation. Columns, % of inhibition of BrdU incorporation as a result of 5 µM MSA treatment. The results are presented as mean ± SE ($n = 3$). *****, $P < 0.05$, statistically different from mock-transfectants.

Overexpression of GKLF induces apoptosis and enhances the effect of MSA on apoptosis induction in PC-3 cells. We next assessed the effect of GKLF overexpression on MSA induction of apoptosis by using the Cell Death Detection ELISA^{PLUS} kit (Roche), which quantitatively detects apoptotic nucleosomes. As shown in Fig. 5, exogenous expression of GKLF in PC-3 cells led to a significant increase of apoptosis. In addition, the effect of MSA on apoptosis induction was greatly enhanced by GKLF overexpression ($P < 0.01$ compared with MSA-treated mock-transfectants).
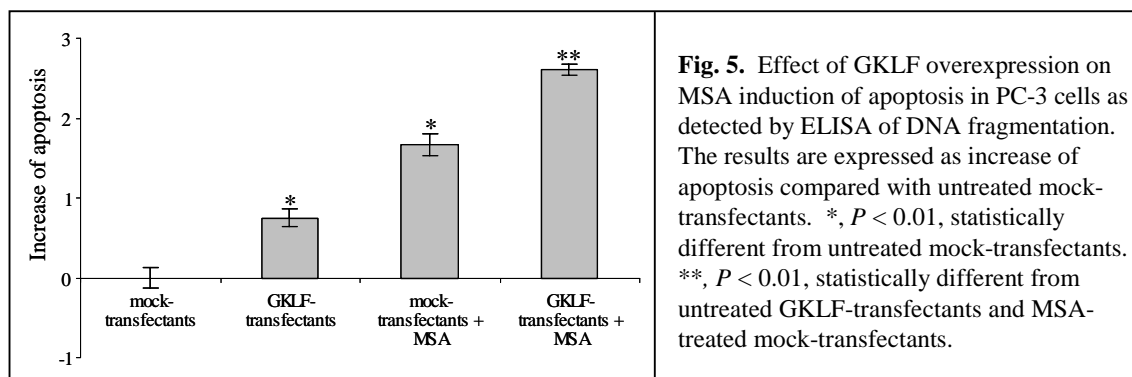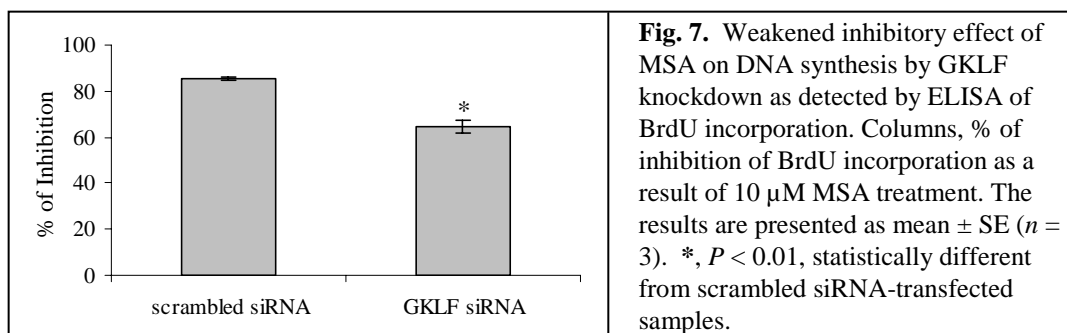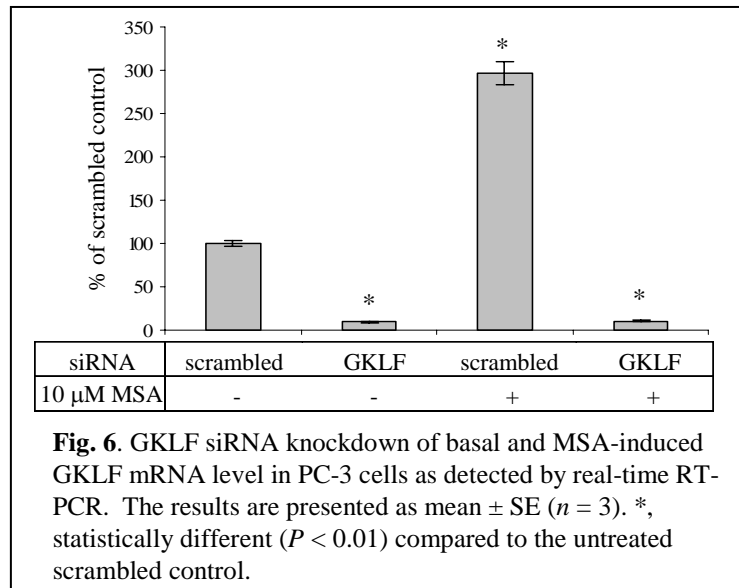

Fig. 5. Effect of GKLF overexpression on MSA induction of apoptosis in PC-3 cells as detected by ELISA of DNA fragmentation. The results are expressed as increase of apoptosis compared with untreated mock-transfectants. *****, $P < 0.01$, statistically different from untreated mock-transfectants. ******, $P < 0.01$, statistically different from untreated GKLF-transfectants and MSA-treated mock-transfectants.

Results for Task 3 (To assess the effect of GKLF gene knockdown on the growth inhibitory and apoptosis inducing actions of selenium):

GKLF knockdown weakens the growth suppressive activity of MSA in PC-3 cells. In order to further establish the role of GKLF induction in mediating MSA action, we adopted the small interference RNA (siRNA) technique to knock down the expression of GKLF. The predesigned Stealth™ Select RNAi for GKLF from Invitrogen was used in this study. The siRNA at a concentration of 40 nM was transiently transfected into PC-3 cells using the Lipofectamine 2000 reagent (Invitrogen). As shown in Fig. 6, the GKLF siRNA transfection not
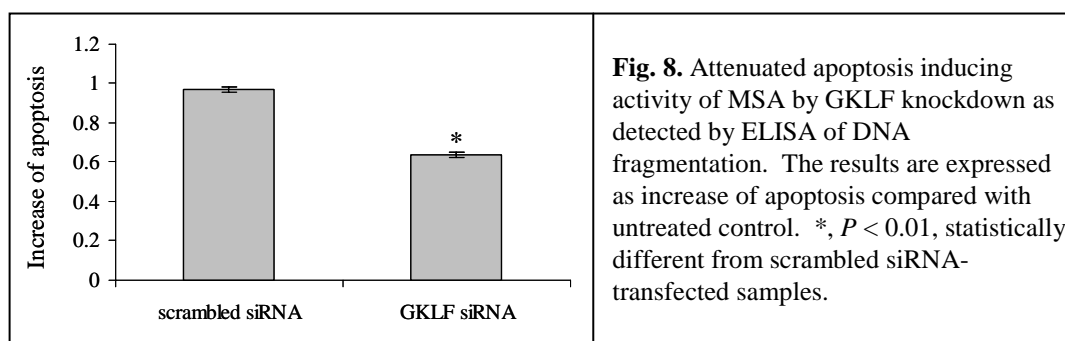
only markedly repressed basal GKLF mRNA expression (~90%), but also prevented the induction of this gene by MSA.

The response of the GKLF knockdown cells to MSA-mediated DNA synthesis inhibition was subsequently examined. The BrdU ELISA assay was performed at 16 hr after 10 µM MSA treatment, and the data are presented in Fig. 7. MSA treatment inhibited DNA synthesis by 73% in the scrambled siRNA-transfected cells, as opposed to 50% in the GKLF siRNA-transfected cells ($P < 0.01$). Thus, GKLF knockdown

siRNA	scrambled	GKLF	scrambled	GKLF
10 µM MSA	-	-	+	+

Fig. 6. GKLF siRNA knockdown of basal and MSA-induced GKLF mRNA level in PC-3 cells as detected by real-time RT-PCR. The results are presented as mean ± SE ($n = 3$). *, statistically different ($P < 0.01$) compared to the untreated scrambled control.

was able to significantly mitigate the growth suppressive activity of MSA.

Fig. 7. Weakened inhibitory effect of MSA on DNA synthesis by GKLF knockdown as detected by ELISA of BrdU incorporation. Columns, % of inhibition of BrdU incorporation as a result of 10 µM MSA treatment. The results are presented as mean ± SE ($n = 3$). *, $P < 0.01$, statistically different from scrambled siRNA-transfected samples.

GKLF knockdown attenuates the apoptosis induction activity of MSA in PC-3 cells. MSA-induced apoptosis was also examined in the GKLF knockdown cells. Without MSA treatment, no difference was observed in apoptosis between transfection with scrambled siRNA or GKLF siRNA (data not shown). In cells treated with 10 µM MSA for 16 hr, MSA-mediated apoptosis induction was greatly attenuated by GKLF gene knockdown ($P < 0.01$). Together with the BrdU data, our results demonstrated an important role of GKLF induction in mediating the growth inhibitory and apoptosis inducing actions of MSA.

Fig. 8. Attenuated apoptosis inducing activity of MSA by GKLF knockdown as detected by ELISA of DNA fragmentation. The results are expressed as increase of apoptosis compared with untreated control. *, $P < 0.01$, statistically different from scrambled siRNA-transfected samples.
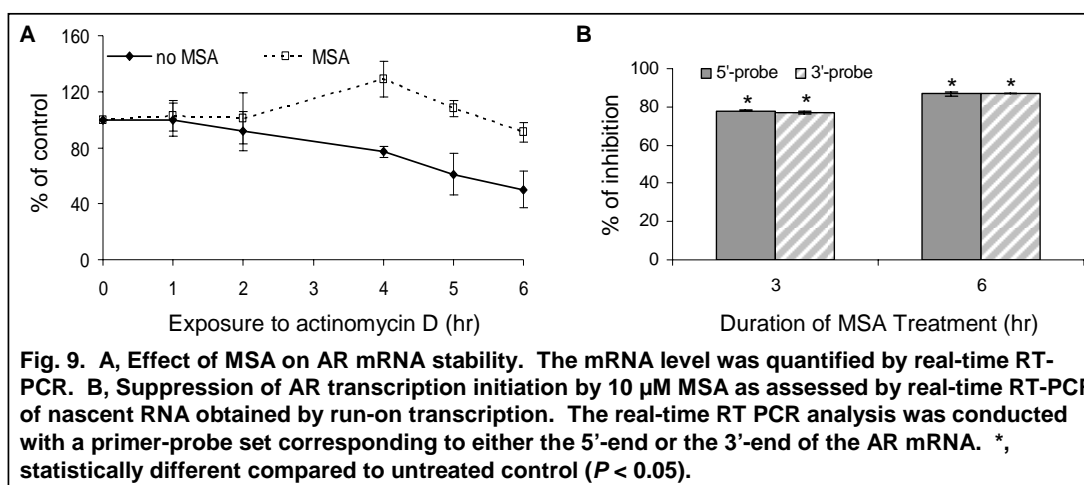
Results for Task 4 (To investigate the effect of androgen receptor overexpression on the growth inhibitory and gene-expression modulating activities selenium):

Please see the attached article (Dong *et al*., Molecular Cancer Therapeutics, 4, 1047-1055, 2005) in Appendix for detailed description of the specific aspects of the research pertinent to this task.

Results for Task 5 (To characterize the mechanism(s) by which selenium suppresses androgen receptor signaling):

<u>**Inhibition of AR transcription initiation by MSA.**</u> To determine whether MSA-mediated downregulation of AR was due to increased mRNA degradation or decreased transcription, we performed an mRNA stability assay. The protocol is the same as described above. Our results showed that treatment with 10 μM MSA actually increased the stability of AR mRNA (Fig. 9A).

Fig. 9. A, Effect of MSA on AR mRNA stability. The mRNA level was quantified by real-time RT-PCR. B, Suppression of AR transcription initiation by 10 μM MSA as assessed by real-time RT-PCR of nascent RNA obtained by run-on transcription. The real-time RT PCR analysis was conducted with a primer-probe set corresponding to either the 5'-end or the 3'-end of the AR mRNA. *, statistically different compared to untreated control ($P < 0.05$).

We then studied the effect of MSA on AR transcription by nuclear run-on assay. Biotin-labeled nascent transcripts obtained by run-on transcription were isolated by using streptavidin particle beads, and quantitated by real-time RT-PCR. To differentiate the effect of MSA on transcription initiation and on transcription elongation, the real-time RT-PCR analysis was conducted with a primer-probe set corresponding to either the 5'-end or the 3'-end of the AR mRNA. For both sets of primer-probe, MSA treatment resulted in ~80% and 87% inhibition of AR transcription at 3 hr and 6 hr, respectively (Fig. 9B). The data thus indicate that the decrease in AR mRNA level by MSA is accounted for by a vigorous block of AR transcription initiation.

<u>**Inhibition of AR promoter activity by MSA.**</u> Gene transcription is generally controlled by promoter regions. In order to study the effect of MSA on AR promoter activity, we PCR amplified an 8 kb and a 1.7 kb fragment of the 5'-flanking region of the human AR gene from an AR-containing BAC clone. The 8 kb fragment (-6885 to +1115) contains ~6.9 kb of the 5'-flanking region upstream of the entire 5'-untranslated region (UTR), and the 1.7 kb fragment (-600 to +1115) contains 600 bp of the 5'-flanking region upstream of the entire 5'-UTR. These two promoter regions were then cloned into the pGL4.19[*luc*2CP/Neo] rapid response luciferase expression vector (Promega). The inclusion of the protein degradation sequences in this vector

allows the reporter to be highly responsive and suitable for monitoring rapid response. The authenticity of the constructs, *pGL4-8kb-AR-promoter and pGL4-1.7kb-AR-promoter*, was confirmed by DNA sequencing. The two constructs were transiently transfected into LNCaP cells cultured in a defined medium (RPMI1640 plus 2% albumax) without androgen to avoid the confounding effect of AR auto-regulation. The treatment duration was 1 hr, at a time when there was no detectable decrease of AR protein. As shown in Fig. 10, MSA induced a ~60% inhibition of the activity

Fig. 10. Suppression of AR promoter activity by 10 µM MSA as assessed by luciferase assay. *, statistically different compared to untreated control ($P < 0.05$).

of both promoters. The data therefore indicate that the *cis* element(s) mediating MSA downregulation of AR is present in the 1.7 kb promoter region. We are in the process of further characterizing such cis-element(s).
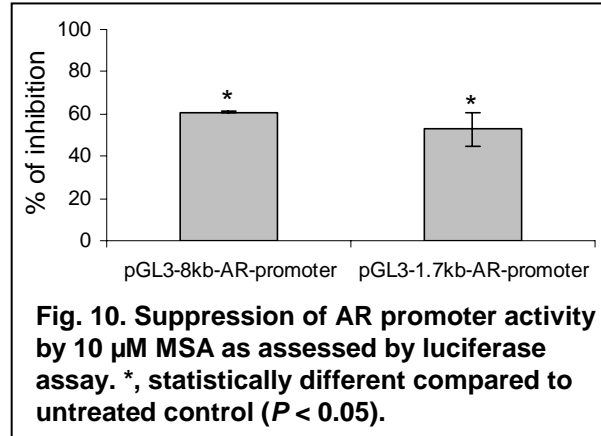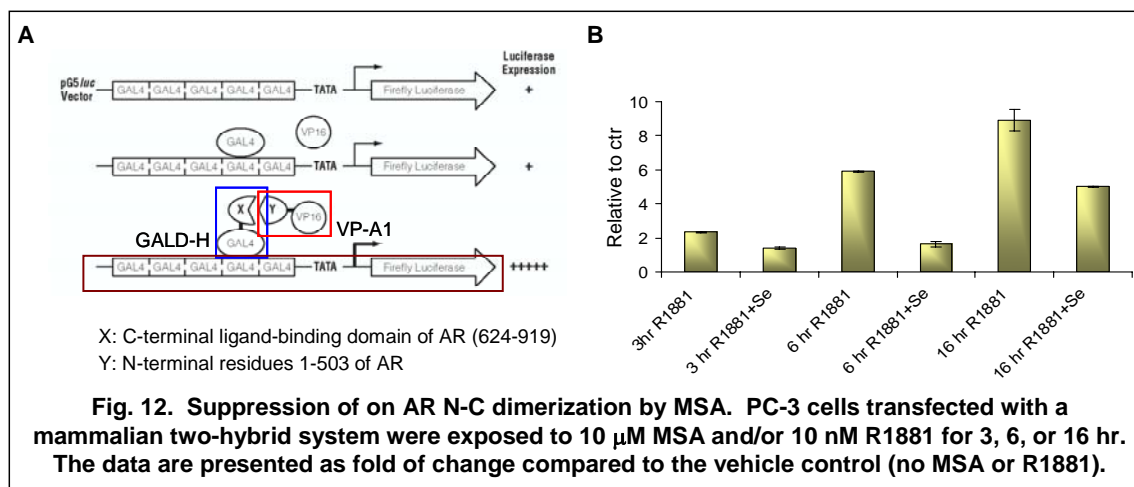
No change of AR ligand-binding by MSA. It is generally believed that unliganded AR localizes in the cytoplasm as a heteromeric complex with heat shock proteins (1, 2). Upon binding to androgens, AR undergoes conformational change, dissociates from the heteromeric partners, forms a homodimer through the interaction of AR N- and C-terminal regions, and translocates into nucleus to initiate target gene regulation (1, 2). In addition, the *trans*-activating activity of AR is modulated by other transcription factors and coregulators (3). Our previous data suggest that MSA may disrupt AR signaling through additional mechanism(s) beyond reducing the availability of the AR protein. We therefore assessed the effect of MSA on the ligand-binding activity of AR.

The ligand-binding activity of AR was analyzed in a whole-cell radioligand binding assay. LNCaP cells were incubated in medium containing charcoal-stripped serum. Increasing concentrations (0.0094, 0.0188, 0.0375, 0.075, 0.15, and 0.3 nM) of [^{3}H] R1881 were added to the culture at the time of 10 µM MSA treatment, with or without a 200-fold molar excess of unlabeled R1881. The radioactivity was measured at the 2-hr time point when there was no change in level of the AR protein. The binding capacity and affinity were determined by Scatchard plot analysis. The values of Bmax (the number of binding sites) and Kd (the concentration of [^{3}H] R1881 producing 50% of total receptor occupancy) are shown in Fig. 11. It is apparent that MSA treatment did not alter the binding capacity or affinity of AR.

Fig. 11. No effect of MSA on AR ligand-binding activity as assessed by the whole-cell radioligand binding assay. LNCaP cells were treated with 10 µM MSA for 2 hr, at a time when there was no change in level of the AR protein. Bmax and Kd represent the number of binding sites and the concentration of [^{3}H] R1881 producing 50% of total receptor occupancy, respectively.
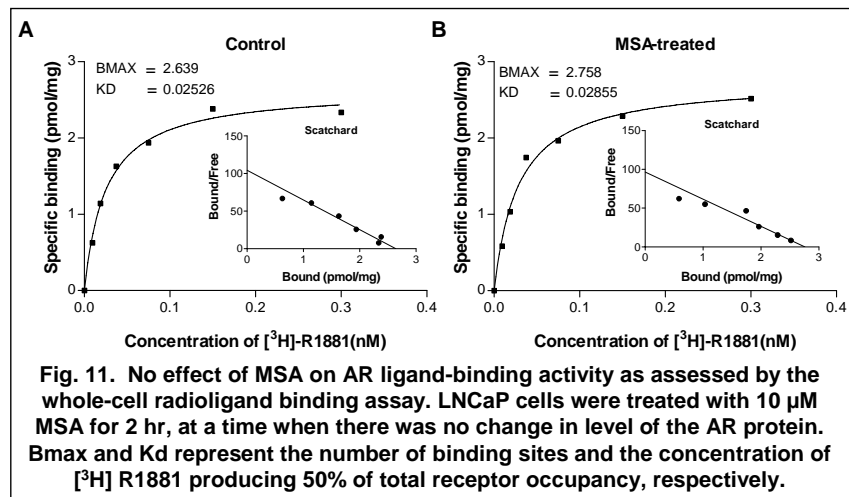
Suppression of AR N-C dimerization by MSA. The effect of MSA on AR N-C dimerization was assessed by using a mammalian two-hybrid system kindly provided by Dr. Elizabeth M. Wilson at the University of North Carolina. As illustrated in Fig. 12A, this system includes two fusion protein constructs, VP-A1 and GALD-H, as well as one reporter gene plasmid, G5E1bLuc (4). VP-A1 is the fusion construct of the N-terminal residues 1-503 of AR and the activation domain of the herpes simplex virus VP16 protein. GALD-H is the fusion construct of the C-terminal ligand-binding domain of AR (624-919) and the GAL4 DNA-binding domain. The G5E1bLuc construct contains the luciferase reporter gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. Once the three constructs are co-transfected into cells, the interaction of the AR N- and C-terminal regions brings together the DNA-binding and transactivation functions, leading to the expression of the reporter gene. Since endogenous AR interferes with the interaction of the two fusion proteins, we transfected the mammalian two-hybrid system into the AR-null PC-3 human prostate cancer cell line. Cells were exposed to 10 µM MSA and/or 10 nM R1881 for 3, 6, or 16 hr. As shown in Fig. 12B, exposure to R1881 resulted in AR N-C dimerization, which was greatly suppressed by MSA. Study is underway to investigate how selenium might alter the folding of AR via redox modification of thiol groups and thereby inhibiting AR N-C dimerization.

X: C-terminal ligand-binding domain of AR (624-919)
Y: N-terminal residues 1-503 of AR

Fig. 12. Suppression of on AR N-C dimerization by MSA. PC-3 cells transfected with a mammalian two-hybrid system were exposed to 10 µM MSA and/or 10 nM R1881 for 3, 6, or 16 hr. The data are presented as fold of change compared to the vehicle control (no MSA or R1881).

C. KEY RESEARCH ACCOMPLISHMENTS:

➢ Selenium upregulates GKLF mRNA level through decreasing GKLF mRNA degradation.

➢ Overexpression of GKLF enhances selenium inhibition of DNA synthesis in the AR-null PC-3 cells.

➢ Overexpression of GKLF induces apoptosis and enhances the effect of selenium on apoptosis induction in PC-3 cells.

➢ GKLF knockdown weakens the DNA synthesis suppressive activity of selenium in PC-3 cells.

➢ GKLF knockdown attenuates the apoptosis induction activity of selenium in PC-3 cells.

➢ Selenium treatment significantly decreases the expression of androgen receptor (AR) and prostate specific antigen (PSA) in five human prostate cancer cell lines (LNCaP, LAPC-

4, CWR22Rv1, LNCaP-C81 and LNCaP-LN3), irrespective of their AR genotype (wild-type *vs.* mutant) or sensitivity to androgen-stimulated growth.

➢ Selenium inhibition of five AR-regulated genes implicated in prostate carcinogenesis (PSA, KLK2, ABCC4, DHCR24, and GUCY1A3) is significantly attenuated by AR overexpression.

➢ Transfection of AR in LNCaP cells weakens significantly the inhibitory effect of selenium on cell growth and proliferation.

➢ Selenium downregulates AR mRNA level through inhibiting AR transcription initiation.

➢ The *cis* element(s) mediating selenium downregulation of AR is present in a 1.7 kb proximal promoter region (-600 to +1115).

➢ Selenium treatment does not alter the ligand-binding capacity or affinity of AR.

➢ Selenium treatment leads to a significant suppression of AR N-C dimerization.

D. REPORTABLE OUTCOMES:

➢ *Publications:*

Dong, Y., Zhang, H., Gao, A.C., Marshall, J.R., and Ip, C. (2005) Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. *Molecular Cancer Therapeutics, 4, 1047-1055.*

Soo O. Lee, Jae Y. Chun, Nagalakshmi Nadiminty, Donald L. Trump, Clement Ip, Yan Dong, and Allen C. Gao. (2006) Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). *Prostate,* 66, 1070-1075.

Haitao Zhang, Yue Wu, Barbara Malewicz, Junxuan Lu, Song Li, James Marshall, Clement Ip, and Yan Dong. (2006) Augmented Suppression of Androgen Receptor Signaling by a Combination of α-Tocopheryl Succinate and Methylseleninic Acid. *Cancer, 107, 2942-2948.*

Shuang Liu, Haitao Zhang, Lijuan Zhao, James Marshall, Xuejian Zhao, and Yan Dong. (2007) Upregulating krüppel-like factor 4 transactivation represents a novel mechanism of selenium growth inhibition. *In preparation.*

➢ *Abstract:*

"Molecular Mechanisms Underlying Selenium Suppression of Androgen Receptor Signaling in Prostate Cancer Cells", 8th International Symposium on Selenium in Biology and Medicine, July, 2006, Madison, Wisconsin.

➢ *Presentations:*

Roswell Park Cancer Institute Chemotherapy/Chemoprevention Research Round, January, 2006, Oral Presentation, "Androgen-Signaling Suppression in Selenium Chemoprevention of Prostate Cancer".

Jilin University, Changchun, P.R. China, June, 2006, Oral Presentation, "Selenium Chemoprevention of Prostate Cancer".

8th International Symposium on Selenium in Biology and Medicine, July, 2006, Poster Presentation, "Molecular Mechanisms Underlying Selenium Suppression of Androgen Receptor Signaling in Prostate Cancer Cells".

> *Funding received:*

NCI Howard Temin (K01) Career Development Award (Dong, PI)   04/05 – 03/10

    o Award per year: $

Jilin Provincial Scholarship for Outstanding Scientists, Jilin Province, P.R. China
(Dong, PI)   02/07 – 01/09

    o Total direct cost: US $

> *Funding applied:*

American Cancer Society Research Scholar Grant (Dong, PI), submitted in October, 2006.

National Cancer Institute P01 (project 3 co-PI, Clement Ip – overall PI, James Marshall – project 3 PI), submitted in June, 2006

> *Employment received:*

Assistant Member, Dept. of Cancer Chemoprevention, Roswell Park Cancer Institute, Buffalo, NY 14263

Member of Graduate Faculty, Graduate Program of Cancer Pathology and Prevention, Roswell Division of Graduate School, State University of New York at Buffalo

Adjunct Professor, Dept. of Pathophysiology, School of Basic Medicine, Jilin University, Changchun, Jilin, P.R. China

E. CONCLUSIONS:

The results from the current study indicate an important role of GKLF induction in mediating the growth suppressive and apoptosis induction activities of selenium in the AR-null PC-3 cells. However, in cells expressing a functional AR, the disruption of AR signaling is most likely more important than the induction of GKLF signaling for selenium action. The vast majority of prostate cancers express a functional AR. Although GKLF has growth suppressive activity in the AR-null cells, such activity might be overshadowed by AR signaling in AR-expressing cells. Therefore, it would be imperative to continue our research effort on the study of selenium suppression of AR signaling.

Almost all patients with advanced prostate cancer respond initially to treatments that interfere with the AR signaling process. However, these treatments often fail after prolonged use and recurrence becomes a major clinical issue (5). The development of hormone refractory prostate cancer is not associated with loss of AR (6, 7). Instead, the accumulation of several

molecular alterations frequently leads to a lower threshold requirement of androgens for the proliferation and survival of prostate cancer cells. Amplification and/or overexpression of AR can hyper-sensitize cells to sub-physiological levels of androgens (8-11). A recent report by Chen *et al.* (8) claimed that increased AR expression is both necessary and sufficient to convert prostate cancer growth from androgen-dependent to -independent, and that AR antagonists may display agonistic activity in cells with elevated AR expression. In addition, AR gene mutations could result in a promiscuous receptor with a broad ligand-binding and *trans*-activation spectrum (12). A selenium intervention strategy aimed at diminishing the expression of AR could be helpful not only for reducing prostate cancer incidence, but also for preventing relapses after endocrine therapy. In addition, the fact that selenium suppresses AR signaling provides a sound rationale for using selenium in combination with an anti-androgen as a new modality for not only the prevention but also the treatment of prostate cancer.

F. List of personnel receiving pay from the research effort

Yan Dong, PhD, PI

Dorothy Donovan, Laboratory Technician

Song Li, Predoctoral Student

G. REFERENCES:

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4   Langley,E., Zhou,Z.X. and Wilson,E.M. Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer, J.Biol.Chem., *270:* 29983-29990, 1995.

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Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers

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Abstract

Our previous report showed that methylseleninic acid (MSA) significantly decreases the expression of androgen receptor and prostate-specific antigen (PSA) in LNCaP cells. The present study extended the above observations by showing the universality of this phenomenon and that the inhibitory effect of MSA on prostate cancer cell growth and cancer-specific biomarkers is mediated through androgen receptor downregulation. First, MSA decreases the expression of androgen receptor and PSA in five human prostate cancer cell lines (LNCaP, LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3), irrespective of their androgen receptor genotype (wild type versus mutant) or sensitivity to androgen-stimulated growth. Second, by using the ARE-luciferase reporter gene assay, we found that MSA suppression of androgen receptor transactivation is accounted for primarily by the reduction of androgen receptor protein level. Third, MSA inhibition of five androgen receptor–regulated genes implicated in prostate carcinogenesis (*PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*) is significantly attenuated by androgen receptor overexpression. Fourth, transfection of androgen receptor in LNCaP cells weakened noticeably the inhibitory effect of MSA on cell growth and proliferation. Androgen receptor signaling has been documented extensively to play an important role in the development of both androgen-dependent and -independent prostate cancer. Our finding that MSA reduces androgen receptor availability by blocking androgen receptor transcription provides justification for a mechanism-driven intervention strategy in using selenium to control prostate cancer progression. [Mol Cancer Ther 2005;4(7):1047–55]

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Introduction

Prostate cancer is the second most common cancer and the second leading cause of cancer death in men in the United States. Androgen plays an important role not only in maintaining the function of the prostate but also in promoting the development of prostate cancer (1). Androgen binds to the androgen receptor, which subsequently translocates to the nucleus and interacts with specific androgen-responsive elements (ARE) on the promoters of target genes. The interaction leads to the activation or repression of genes involved in the proliferation and differentiation of the prostate cells (2). Prostate-specific antigen (PSA) and kallikrein 2 (KLK2) are two well-accepted targets of androgen receptor. PSA, also known as kallikrein 3, is an established serum marker for the diagnosis and prognosis of prostate cancer. Although KLK2 is not as widely used as PSA, it is increasingly recognized to provide added information to disease staging (3, 4).

The randomized, placebo-controlled Nutritional Prevention of Cancer trial showed that selenium supplementation reduced the incidence of prostate cancer by 50% (5, 6). This trial was designed initially to assess the effect of selenium on nonmelanoma skin cancer. Because men accounted for a sizable proportion of the cohort (974 of a total of 1,312), there was sufficient power to analyze the changes in prostate cancer risk. When the prostate cancer data were further stratified, there was evidence of a greater reduction in risk from selenium supplementation among men who had low baseline plasma PSA levels (6). Early-stage prostate cancer is mostly responsive to androgen stimulation. The inference that the protective effect of selenium might be more pronounced in early-stage prostate cancer, as reflected by low PSA secretion, lends credence to the idea that selenium might affect androgen signaling.

Recently, we reported that a selenium metabolite, in the form of methylseleninic acid (MSA), greatly down-regulates the expression of androgen receptor and PSA in the androgen-responsive LNCaP human prostate cancer cells (7, 8). The suppression of androgen receptor signaling occurs well before any significant growth inhibition, which is accompanied by correlative changes in numerous cell

cycle and apoptosis regulatory molecules (9–13). Androgen receptor signaling involves multiple steps, the receptor itself is just one of many effectors that participate in the process. For example, heat shock proteins are known to modulate the stability of androgen receptor as well as its affinity to androgen (14, 15). The trans-activating activity of androgen receptor can be affected markedly by a large number of coactivators and corepressors (16). Our microarray analysis suggests that MSA alters the expression of several heat shock proteins, coactivators, and corepressors of the superfamily of steroid hormone receptors (17). In view of these confounding effects, the present study was designed to determine the role of androgen receptor down-regulation per se in MSA interference of androgen receptor signaling. Our approach was to use the ARE-luciferase reporter gene assay to find out the extent to which selenium suppression of androgen receptor transactivation could be reversed when the luciferase activity is normalized based on androgen receptor protein level. We also investigated whether androgen receptor transfection might attenuate selenium-mediated down-regulation of five androgen receptor targets: *PSA*, *KLK2*, ATP-binding cassette C4 (*ABCC4*, also known as *MRP4*), 24-dehydro-cholesterol reductase (*DHCR24*, also known as *seladin-1*), and soluble guanylate cyclase 1 α 3 (*GUCY1A3*). These five androgen-inducible genes were selected based on the criteria that they are consistently overexpressed in prostate cancer compared with normal prostate tissue (18). Finally, in an effort to evaluate the biological significance of the selenium-androgen receptor signaling axis, we investigated whether androgen receptor over-expression might block the growth inhibitory effect of selenium.

Materials and Methods

Selenium Reagent, Prostate Cancer Cell Lines, 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay, and Bromodeoxyuridine-Labeling Analysis

MSA was synthesized as previously described (19). The LNCaP and CWR22Rv1 human prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). The LAPC-4 cell line was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center. The two androgen-unresponsive LNCaP sublines, LNCaP-LN3 and LNCaP-C81, were obtained from Dr. Curtis A. Pettaway (University of Texas M.D. Anderson Cancer Center) and Dr. Ming-Fong Lin (University of Nebraska Medical Center), respectively. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell growth and the bromodeoxyuridine (BrdUrd) labeling for DNA synthesis were done as described in our previous publication (17).

Transient Transfection of Androgen Receptor

The procedure was carried out using the Lipofect-AMINE Plus reagent (Invitrogen, Carlsbad, CA) per instruction of the manufacturer. At 24 hours before transfection, cells were plated in growth medium without antibiotics at a density to reach 90% to 95% confluency at transfection. The pSG5hAR androgen receptor expression vector (20) or the pSG5 mock plasmid (Stratagene, La Jolla, CA) was introduced into LNCaP cells with or without the cotransfection of the pEGFP-F membrane-GFP-encoding construct (BD Biosciences, San Jose, CA). The purpose of the green fluorescent protein (GFP) was to enable us to enrich for the subset of positively androgen receptor–transfected cells. During cotransfection, the two plasmids were added at 1:1 molar ratio. The amount of DNA transfected was 12 μg per 10-cm culture dish. The DNA/liposome mixture was removed at 3 hours after transfection. For the MTT assay, the cells were trypsinized 16 hours later and plated in triplicate onto a 96-well plate. Cells were allowed to recover for an additional 24 hours before exposure to 10 μmol/L MSA. The MTT assay was conducted at 48 hours post-MSA treatment. For the BrdUrd-labeling analysis, the cells were subjected to MSA treatment at 24 hours posttransfection and labeled with BrdUrd after 24 hours of MSA treatment.

Reporter Gene Assay

The ARE-luciferase reporter plasmid, containing three repeats of the ARE region ligated in tandem to the luciferase reporter (20), was transiently transfected into cells at a concentration of 9 μg per 10-cm culture dish. After incubating with the transfection mixture for 3 hours, the cells were trypsinized, resuspended in medium containing charcoal-stripped serum and 10 nmol/L dihydrotestosterone (Sigma, St. Louis, MO), and plated in triplicate onto 6-well plates. Cells were allowed to recover for an additional 24 hours before exposure to 10 μmol/L MSA. After 6 or 16 hours of MSA treatment, cells were lysed in reporter lysis buffer (Promega, Madison WI), and the luciferase activity was assayed using the Luciferase Assay System (Promega). Protein concentration in cell extracts was determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized by the protein concentration of the sample. The transfection experiments were repeated thrice.

Western Blot Analysis

Details of the procedure for Western blot analysis were described previously (17). Immunoreactive bands were quantitated by volume densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase. The following monoclonal antibodies were used in this study (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti–androgen receptor (BD Biosciences), and anti-PSA (Lab Vision, Fremont, CA).

Real-time Reverse Transcription-PCR

Real-time reverse transcription-PCR analysis was done as described previously (21). The PCR primers and

Taqman probes for β-actin, androgen receptor, PSA, KLK2, ABCC4, DHCR24, and GUCY1A3 were Assays-on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 minutes, then a denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantitation of gene expression was done using the comparative C_T ($\Delta\Delta C_T$) method (22).

Androgen Receptor mRNA Stability Assay

Actinomycin D (5 μg/mL) was added to the cultures to stop new RNA synthesis at the time of MSA treatment, and androgen receptor mRNA levels were measured by real-time reverse transcription-PCR at hourly intervals for the next 6 hours. The turnover of androgen receptor mRNA was determined by comparing mRNA levels over time in cells treated with or without MSA.

Statistical Analysis

The Student's two-tailed t test was used to determine significant differences between treatment and control values, and $P < 0.05$ was considered statistically significant.

Results

MSA Depresses Androgen Receptor Transcription

Figure 1 shows the effect of MSA on androgen receptor transcript and protein levels as well as androgen receptor mRNA stability in LNCaP cells. The decrease in androgen receptor transcript, as determined by real-time reverse transcription-PCR, occurred very quickly (Fig. 1A). On the average, there was about a 50% reduction in the first three hours after treatment with 10 μmol/L MSA; by 6 hours, the magnitude of inhibition rose to 80%. At the protein level, there was no change in androgen receptor in the first two hours (Fig. 1B). A modest decrease began to appear at 3 hours, and the inhibition became very pronounced at 6 hours (Fig. 1B). The observation is consistent with the time-dependent sequence of reduced mRNA leading to decreased protein expression. To determine whether the down-regulation of androgen receptor mRNA was due to decreased transcription or increased mRNA degradation, we did an mRNA stability assay under the condition in which new RNA synthesis was blocked. Actinomycin D was added to the culture at the time of MSA treatment, and androgen receptor mRNA levels were followed in a 6-hour time course experiment. Because actinomycin D could be cytotoxic, we also monitored cell growth for up to 8 hours and did not observe cell death or significant growth inhibition during this period. Our results showed that treatment with MSA actually increased the stability of androgen receptor mRNA (Fig. 1C). This observation rules out increased mRNA degradation as a contributing factor. Therefore, the decrease in androgen receptor mRNA level by MSA is likely to be accounted for by a vigorous block of androgen receptor transcription.

We next examined the effect of MSA on the expression of androgen receptor and PSA in four additional human prostate cancer cell lines: LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3. The LAPC-4 cells are androgen responsive and express a wild-type androgen receptor (23), as opposed to LNCaP cells that are also androgen responsive but express a mutant, although functional, androgen receptor. The other three cell lines are all androgen-unresponsive and express a mutant but functional androgen receptor (24–27). As shown in Fig. 2 (*left*), MSA decreased androgen receptor and PSA transcript levels progressively as a function of time in all four cell lines examined. The reduction in androgen receptor and PSA proteins (*right*) paralleled the drop in the transcripts. In LAPC-4, CWR22Rv1, and LNCaP-C81 cells, a decrease in PSA transcript was already detectable as early as 3 hours, at a time when there was no apparent loss of the androgen receptor protein. The data suggest that MSA disrupts androgen receptor signaling through additional mechanism(s) beyond reducing the availability of the androgen receptor protein.
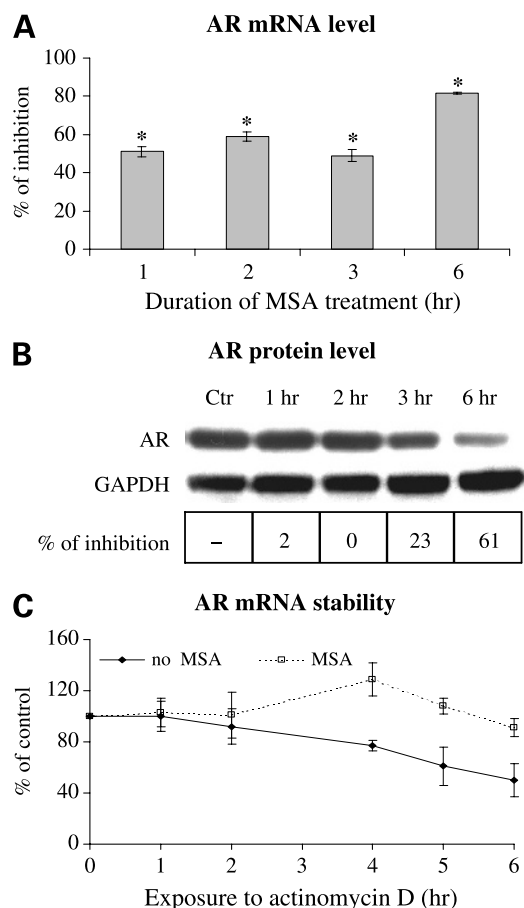

Figure 1. Effect of MSA on androgen receptor (*AR*) expression in LNCaP cells. **A,** inhibition of androgen receptor mRNA level as determined by real-time RT-PCR. **B,** inhibition of androgen receptor protein level as determined by Western blot analysis. **C,** androgen receptor mRNA stability in the presence or absence of MSA. *Bars,* SE. *, $P < 0.05$, statistically different compared with untreated control.
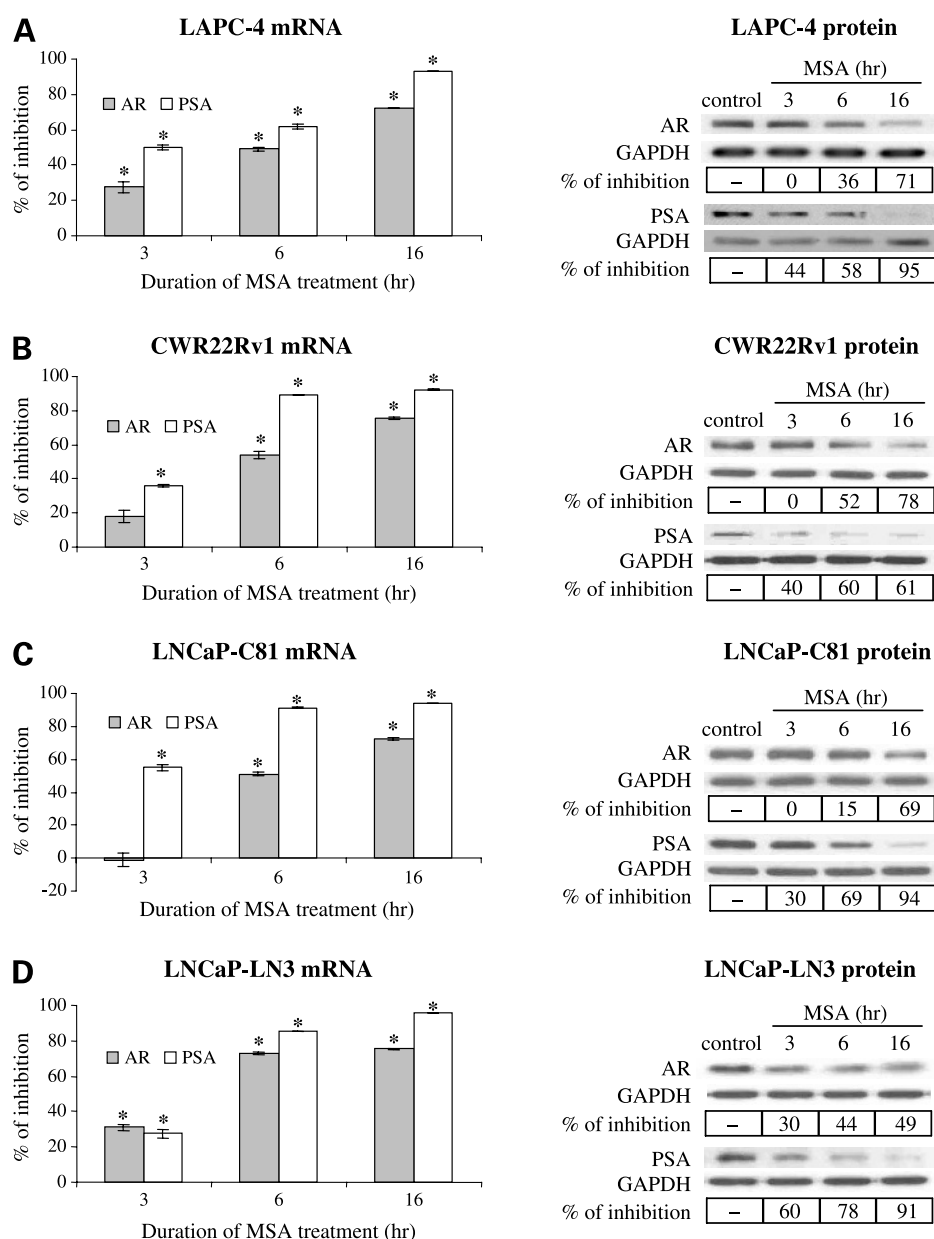
Figure 2. Effect of MSA on androgen receptor (*AR*) and PSA expression in LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3 cells. *Left,* mRNA levels as determined by real-time RT-PCR; *right,* protein levels as determined by Western blot analysis. *Columns,* % inhibition; *bars,* SE. *, *P* < 0.05, statistically different compared with untreated control.

MSA-Mediated Androgen Receptor Down-Regulation Leads to a Reduction of Androgen Receptor Transactivating Activity

The transactivation of androgen receptor is an indicator of androgen receptor signaling and can be quantified readily by a reporter gene assay. To investigate whether the reduced availability of androgen receptor by MSA is a major factor in modulating androgen receptor transcriptional activity, we transiently transfected LNCaP cells with the ARE-luciferase reporter plasmid and normalized the luciferase activity based on the level of the androgen receptor protein. This normalization step eliminates the level of androgen receptor expression as a determinant of androgen receptor transactivation. The luciferase reporter assay was carried out at 6 and 16 hours after treatment with 10 μmol/L MSA. At these two time points, androgen receptor protein level was inhibited by 60% and 77%, respectively (Fig. 3B, *inset*). As can be seen in Fig. 3A, without normalizing for the difference in androgen receptor protein level between the MSA-treated and -untreated samples, the ARE-promoter activity was decreased by 65% or 75%, respectively, after 6 or 16 hours of MSA treatment. However, after normalization, the ARE-promoter activity was inhibited by a meager 15% at the 6-hour time point, and the inhibition disappeared completely at 16 hours (Fig. 3B). These findings suggest that the reduced availability of the androgen receptor protein is the major factor in contributing to MSA disruption of androgen receptor signaling.

Overexpression of Androgen Receptor Attenuates the Effect of MSA on the Down-Regulation of Androgen Receptor–Regulated Genes

To delineate the role of low androgen receptor abundance as a cause of reduced PSA expression by selenium, we transiently transfected LNCaP cells with a wild-type androgen receptor construct and determined the response of PSA to MSA. After 3 hours of MSA exposure, PSA transcript was depressed by about 75% in the mock-transfected cells but only by about 45% in the androgen receptor–transfected cells (Fig. 4A). Based on our routine experience of a 40% transfection efficiency as determined by GFP cotransfection analysis (described below), we believe that the inhibitory effect of MSA on PSA mRNA might have been reversed completely in positive androgen receptor transfectants. Our conclusion was derived from the following theoretical calculation: 40% of $(1 - x) + 60\%$ of $(1 - a) = 1 - b$, where $x = \%$ inhibition in positive androgen receptor transfectants, $a = 75\%$ inhibition in mock transfectants, and $b = 45\%$ inhibition in the mixed population of androgen receptor–transfected cells. Solving for x in the above equation gave a value of 0% inhibition. In
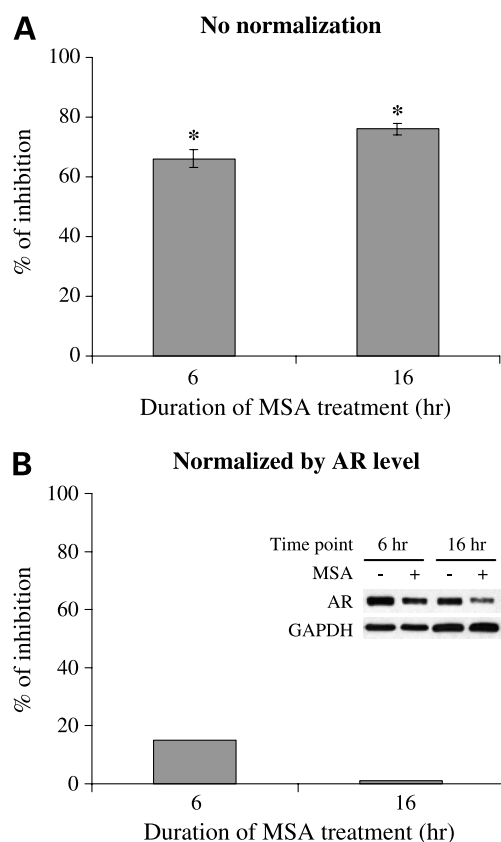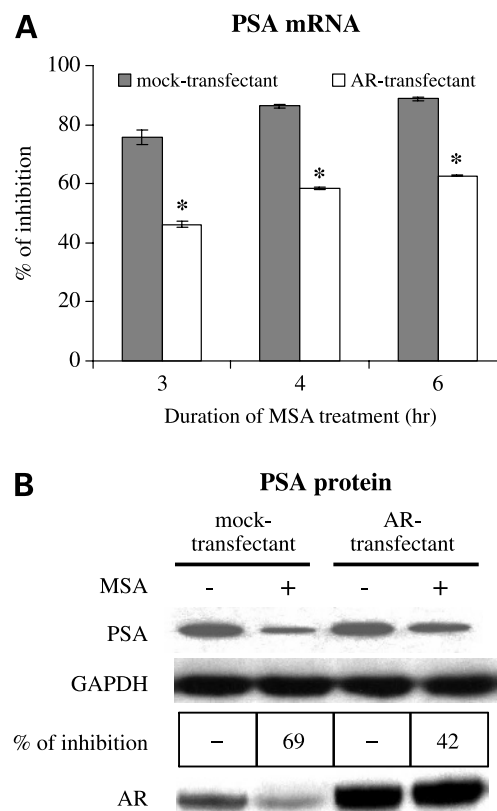
A PSA mRNA

Figure 4. Effect of androgen receptor (*AR*) overexpression on MSA down-regulation of PSA. **A,** PSA mRNA expression as determined by real-time RT-PCR, in androgen receptor– or mock-transfected LNCaP cells treated with MSA. *Columns,* % inhibition. *, *P* < 0.05, statistically different compared with the value from the mock transfectant. **B,** PSA Western blot analysis in androgen receptor– or mock-transfected LNCaP cells treated with MSA.

other words, there was no inhibition of PSA expression by MSA in the positive androgen receptor transfectants (i.e., complete reversal). The difference between the mock- and androgen receptor–transfected cells, although still apparent, was not as great at 4 and 6 hours compared with that at 3 hours. The fact that a robust androgen receptor presence was not sufficient to completely counteract the suppressive effect of MSA on the transcription of PSA at the later time points suggests that there could be a delay in the recruitment of additional mechanisms by which MSA might diminish androgen receptor signaling. We also studied the protein level of PSA by Western blotting. The Western analysis was done at 24 hours after MSA treatment. As shown in Fig. 4B, PSA protein was depressed by about 70% in the mock-transfected cells but only by about 40% in the androgen receptor–transfected cells. The protein levels of androgen receptor in the mock- and androgen receptor–transfected cells are also shown in Fig. 4B for confirmation purposes.

Using a bioinformatic data mining approach, we recently identified five additional androgen-inducible genes that are expressed at a higher level in prostate cancer compared

Figure 3. Effect of MSA on ARE-promoter activity before (**A**) and after (**B**) normalizing by androgen receptor (*AR*) protein level. **B,** representative Western blot analysis of androgen receptor protein level in the cell extracts (*inset*). Androgen receptor protein level was depressed by 60% or 77% at 6 or 16 h, respectively. *Columns,* % inhibition; *bars,* SE. *, *P* < 0.05, statistically different compared with untreated control.

with normal prostate tissue; furthermore, their expression is repressed by MSA (18). These genes are *KLK2*, *ABCC4* (also known as *MRP4*), *DHCR24* (also known as *seladin-1*), *GUCY1A3*, and long-chain fatty acid CoA ligase 3 (*FACL3*). MSA down-regulation of their expression only occurs in LNCaP cells but not in the androgen-unresponsive PC-3 cells that express an extremely low level of androgen receptor (18). To verify that the decreased expression of these genes is a direct consequence of MSA suppression of androgen receptor signaling, we applied the same androgen receptor overexpression protocol as described above and used real-time reverse transcription-PCR to quantitate their transcript levels. The *FACL3* gene was not included in this study as no Assays-on-Demand primers and probes are available for this gene. The results are shown in Fig. 5. Androgen receptor transfection significantly muted the inhibition of gene expression by MSA. In general, the difference in % inhibition between the mock and androgen receptor transfectants was greatest at 3 hours and narrowed gradually with time. The overall pattern was very similar for *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. The data thus show a key role of androgen receptor down-regulation in mediating the inhibitory effects of MSA on the expression of androgen receptor–regulated genes.

Overexpression of Androgen Receptor Interferes with MSA-Mediated Growth Inhibition

In an effort to evaluate the biological significance of MSA suppression of androgen receptor signaling, we transiently transfected LNCaP cells with a wild-type androgen receptor and assessed the response of the androgen receptor–overexpressing cells to MSA-induced growth inhibition. The MTT assay was conducted at 48 hours post-MSA, and the data are presented in Fig. 6A. In the absence of MSA, cell growth was not altered by the transfection of androgen receptor (data not shown), indicating that the endogenous level of androgen receptor is not a limiting factor for the growth of these cells. MSA treatment inhibited growth by 40% in the mock transfectants, as opposed to 27% in the androgen receptor transfectants. The difference is statistically significant ($P = 0.003$). Thus, androgen receptor overexpression was able to weaken the growth suppressive activity of MSA. One reason that the difference was seemingly compressed was due to the fact that only a fraction of cells was successfully transfected, and in this study, cell growth was assessed using the whole cell population. To address the last problem, we cotransfected cells with the androgen receptor expression vector and a membrane-GFP-encoding construct. The cells were then subjected to BrdUrd labeling, and the data were analyzed by gating just the GFP-positive cells. As shown in Fig. 6B, after selecting for the subset of GFP-positive cells, we found that MSA inhibited DNA synthesis by a very modest 16% in the androgen receptor transfectants, as opposed to 72% in the mock transfectants. Because the GFP and androgen receptor cDNAs are not located in the same plasmid construct, it is possible that not all the cells positive for GFP are also positive for the transfected androgen receptor. Thus, our selection process only led to an enrichment, rather than an exclusive selection, of double-positive cells. Therefore, the difference
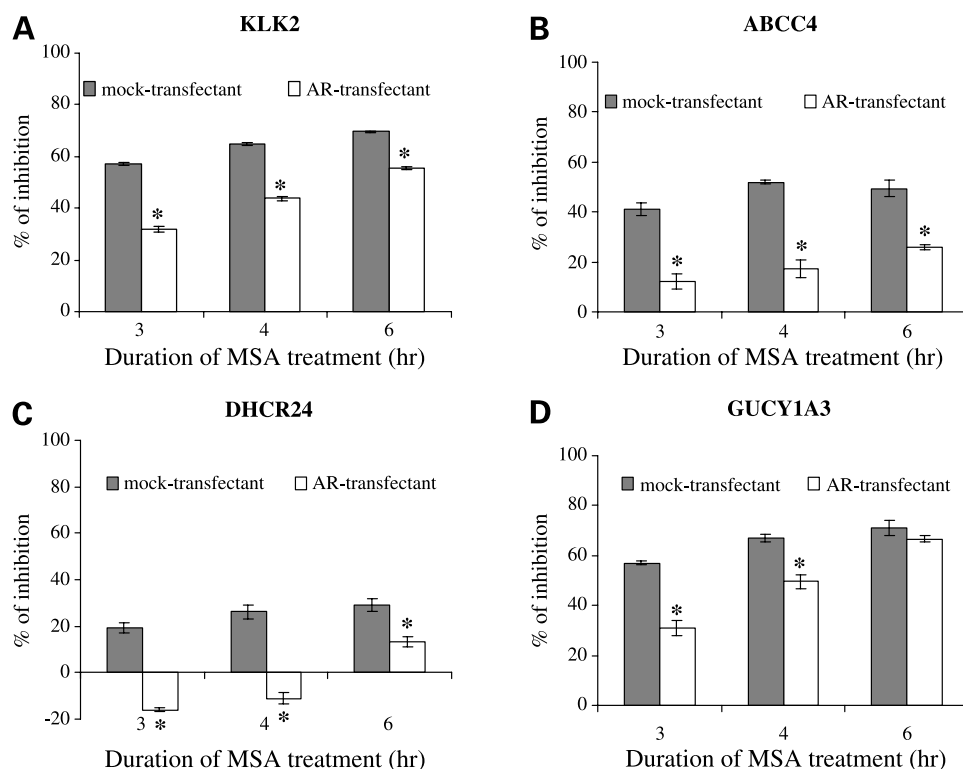

Figure 5. Effect of androgen receptor (*AR*) overexpression on MSA down-regulation of KLK2, ABCC4, DHCR24, and GUCY1A3 mRNA expression as determined by real-time RT-PCR, in androgen receptor– or mock-transfected LNCaP cells treated with MSA. *Columns,* % inhibition. *, *P* < 0.05, statistically different compared with the value from the mock transfectant.
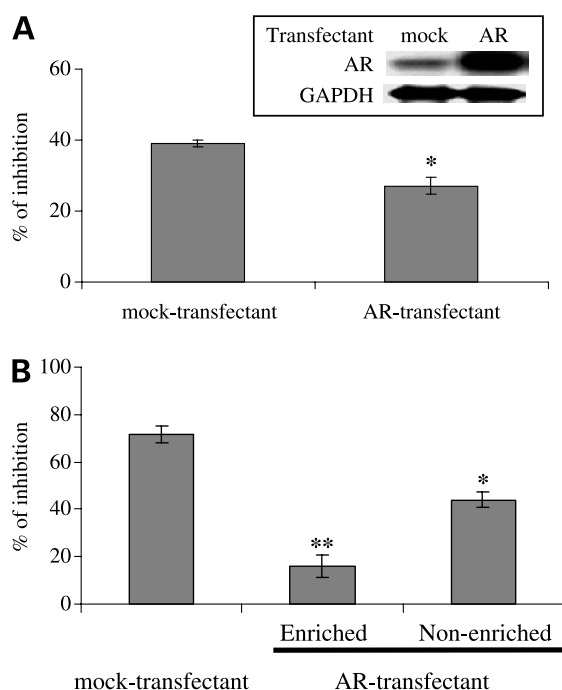
A

B

Figure 6. Effect of androgen receptor (*AR*) overexpression on MSA inhibition of cell growth. **A,** MTT cell growth assay in androgen receptor– or mock-transfected LNCaP cells treated with MSA. Western blot confirmation of androgen receptor protein level (*inset*). **B,** BrdUrd labeling of selected GFP-positive or nonselected androgen receptor–transfected LNCaP cells treated with MSA. *Columns,* % inhibition compared with untreated control. *, *P* < 0.05, statistically different from mock trans-fectant. **, *P* < 0.05, statistically different from mock transfectant and nonselected androgen receptor transfectant.

between the mock transfectants and the androgen receptor transfectants might have been even more pronounced if all the cells used in the experiment were successfully transfected with androgen receptor. Figure 6B also shows that when we did the BrdUrd labeling experiment with the nonenriched androgen receptor–transfected cells, the inhibition by MSA was about 45%, a value half-way between that achieved by the mock transfectants and the enriched androgen receptor transfectants.

Discussion

Our previous report showed that selenium significantly decreases the expression and the transactivating activity of androgen receptor in LNCaP cells (21). The present study extended the above observations by showing the universality of this phenomenon and a key role of androgen receptor down-regulation in mediating the inhibitory effects of selenium on prostate cancer cell growth and the expression of cancer-specific biomarkers. First, selenium decreases the expression of androgen receptor and PSA in five human prostate cancer cell lines, irrespective of their androgen receptor genotype (wild type versus mutant) or sensitivity to androgen-stimulated growth. Second, a reporter gene assay with the ARE-luciferase construct indicated that depletion of the androgen receptor protein

is a major factor for selenium depression of androgen receptor transactivating activity. Third, overexpression of androgen receptor greatly weakens the inhibitory effects of selenium on prostate cancer cell proliferation as well as the expression of five androgen receptor–regulated genes implicated in prostate carcinogenesis: *PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. These findings, however, do not necessarily exclude additional mechanisms by which selenium diminishes androgen receptor signaling (e.g., via modulation of ligand binding, androgen receptor dimerization, nuclear translocation, and the interaction of androgen receptor with its coregulators). In fact, our previous report provided some evidence that selenium is able to inhibit the binding of androgen receptor to the ARE in the absence of a drop in the androgen receptor level (21).

A selenium intervention strategy aimed at diminishing the expression of androgen receptor could be helpful not only for reducing prostate cancer incidence but also for preventing relapses after endocrine therapy. Almost all patients with advanced prostate cancer respond initially to treatments that interfere with the androgen receptor–signaling process. However, these treatments often fail after prolonged use and recurrence becomes a major clinical issue (28). The development of hormone refractory prostate cancer is not associated with loss of androgen receptor (29, 30). Instead, the appearance of several molecular alterations frequently leads to a lower threshold requirement of androgens for the proliferation and survival of prostate cancer cells. Androgen receptor gene mutations could result in a promiscuous receptor with a broad ligand-binding and transactivation spectrum (31). Amplification and/or overexpression of androgen receptor may hyper-sensitize cells to subphysiologic levels of androgens (32–35). A recent report by Chen et al. (35) claimed that increased androgen receptor expression is both necessary and sufficient to convert prostate cancer growth from androgen-dependent to -independent and that androgen receptor antagonists may display agonistic activity in cells with elevated androgen receptor expression. On the other hand, several studies showed that knocking down the expression of androgen receptor inhibits the growth of prostate cancer cells, both *in vitro* and *in vivo*, and induces apoptosis (36–39). Because selenium blocks the transcription of androgen receptor (see Fig. 1), this treatment modality may prove to be effective in prostate cancer intervention.

The down-regulation of androgen receptor targets by selenium has important clinical implication. We have studied *PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. All these genes are expressed at a higher level in prostate cancer compared with normal prostate tissue (18). PSA and KLK2 are prostate-specific differentiation markers. They belong to the serine protease family and are both secretory proteins. PSA is the most useful serum marker for the diagnosis and prognosis of prostate cancer. The combined use of PSA and KLK2 has been shown to improve the specificity of biochemical detection of prostate cancer (40–44) and the accuracy in predicting tumor grade and

stage (3, 4). ABCC4 (also known as MRP4) is a member of the multidrug resistance-associated protein family of transporters. Overexpression of ABCC4 in neuroblastoma is associated with poor prognosis and resistance to the topoisomerase I poison irinotecan and its active metabolite SN-38 (45). Thus, the down-regulation of MRP4 by selenium might represent a potential mechanism by which selenium enhances the therapeutic efficacy of a number of anticancer drugs, including irinotecan (46). DHCR24 (also known as seladin-1) is an antiapoptotic protein, it inhibits the activity of caspase 3 (47). The overexpression of this gene has also been reported in adrenocortical adenoma cells compared with adjacent nontumor cells (48). GUCY1A3 catalyzes the conversion of GTP to the second messenger cyclic guanosine $3',5'$-monophosphate, which regulates the activity of protein kinases, phosphodiesterases, and ion channels (49). Future selenium intervention trial may consider monitoring androgen receptor, PSA, KLK2, ABCC4, DHCR24, and GUCY1A3 in biopsied prostate samples, to obtain a more comprehensive picture of an individual's responsiveness to selenium. Recent data also showed that cellular PSA is more sensitive than secretory PSA to selenium intervention (50). This is one more reason why it is preferable to do the analysis in biopsied prostate tissue.

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